



## Short communication

# *In vitro* inhibition and *in vivo* induction of defense response against *Penicillium expansum* in sweet cherry fruit by postharvest applications of *Bacillus cereus* AR156



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## ARTICLE INFO

## Article history:

Received 2 September 2014

Received in revised form 9 November 2014

Accepted 9 November 2014

## Keywords:

*Prunus avium*

Biocontrol

Blue mold

Mechanism of action

Disease resistance

## ABSTRACT

The biocontrol effect of *Bacillus cereus* AR156 on blue mold decay caused by *Penicillium expansum* in harvested sweet cherry fruit and the possible mechanisms were investigated. The results indicated that *B. cereus* AR156 treatment significantly reduced disease incidence and development. The treatment significantly enhanced activities of chitinase and  $\beta$ -1, 3-glucanase in the fruit. *B. cereus* AR156 damaged the plasma membrane integrity of *P. expansum* spores and caused the leakage of protein and sugar of the pathogen mycelia *in vitro*. The results indicated that the efficacy of *B. cereus* AR156 on controlling blue mold decay in cherry fruit may be related to the direct fungitoxic property against the pathogen, and the induction of defense-related enzymes in the fruit.

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## 1. Introduction

Sweet cherry (*Prunus avium* L.) is susceptible to postharvest decay caused by several pathogenic fungi. Blue mold decay caused by *P. expansum*. Link is one of the most important postharvest diseases of sweet cherries (Ceponis et al., 1987). Traditionally, control of postharvest diseases of fruit relies mainly on the application of synthetic fungicides. However, fungicide resistance of pathogens and public concern over chemical residues in food and environment have created interest in alternative approaches to disease control (Janisiewicz and Korsten, 2002).

Biological control using microbial antagonists has emerged as an effective strategy to suppress postharvest diseases of fruit and vegetables (Sharma et al., 2009). It has been reported that *Pichia caribbica* reduced blue mold decay caused by *P. expansum* in apples (Cao et al., 2013). *Pichia membranefaciens* has also been shown effective in controlling postharvest diseases of sweet cherry fruit (Qin et al., 2006). Recently, *Bacillus subtilis* has been evaluated as a potential biocontrol agent against postharvest pathogenic fungi in various fruit (Zhou et al., 2011; Wang et al., 2013; Waewthongrak et al., 2015). However, there is no information concerning the

biocontrol effect of *B. cereus* AR156 on blue mold decay in sweet cherry fruit. Thus, the objective of this study was to evaluate the efficacy of *B. cereus* AR156 for the control of blue mould caused by *P. expansum* in cherry fruit and to explore the possible mechanisms involved.

## 2. Materials and methods

## 2.1. Antagonist and fungal pathogen

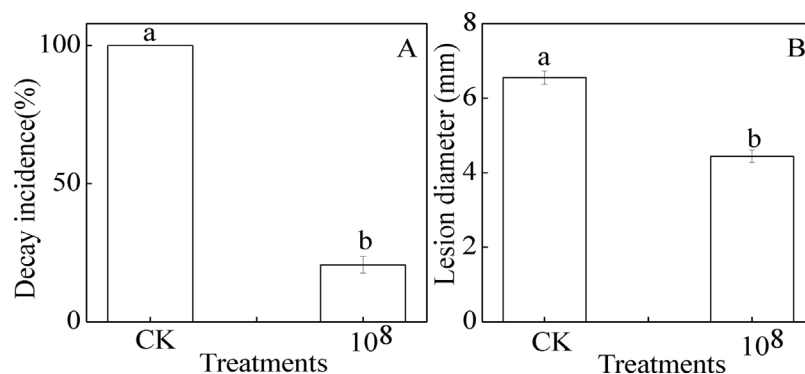
The biocontrol antagonist, *B. cereus* AR156 was kindly supplied by Prof. Jianhua Guo of College of Plant Protection, Nanjing Agricultural University, China. The bacterial strain was cultured on nutrient agar medium at 37 °C for 24 h. Cells were harvested by centrifugation at 5000 × g for 5 min and suspended in sterile distilled water. The cell concentration was adjusted to 1 × 10<sup>8</sup> CFU/mL. The pathogen *P. expansum* was isolated from infected sweet cherry fruit and cultured on potato dextrose agar medium. Spore concentration was adjusted to 5 × 10<sup>4</sup> CFU/mL with sterile distilled water containing 0.05% (v/v) Tween 80.

## 2.2. Fruit and treatments

Sweet cherry (*Prunus avium* L. cv. Hongdeng) fruit were hand-harvested at the commercial maturity stage with healthy greenish

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**Fig. 1.** Effects of *B. cereus* AR156 on decay incidence (A), lesion diameter (B) in sweet cherry fruit. Data are expressed as the mean of triplicate samples. Bars represent standard deviations of the means. Data in columns with the different letters are significantly different according to Duncan's multiple range tests at  $p < 0.05$ .

stems, from an orchard in Yantai, Shandong province, China. Fruit was selected for uniformity of size, ripeness, and absence of defects. Fruit was disinfected with 2% (v/v) sodium hypochlorite for 2 min, washed with tap water, and air-dried prior to wounding. Sweet cherries were wounded (3 mm deep and 3 mm wide) with a sterile nail. Aliquots (20  $\mu$ L) of antagonist at  $1 \times 10^8$  CFU/mL or distilled water (as the control) were pipetted into each wound. Three hours later, each wound was inoculated with 15  $\mu$ L of a suspension of  $5 \times 10^4$  spores per mL pathogen. The fruit were incubated at 20°C and 95% RH for 5 days. Disease incidence and lesion diameter were recorded after storage. Fruit samples were taken from 10 fruit for enzyme assays at 0, 1, 2, 3, 4 and 5 days.

### 2.3. Effects of *B. cereus* AR156 on plasma membrane integrity, protein and sugar leakage of *P. expansum* spores and enzyme activities of sweet cherry fruit

All enzyme extract procedures were conducted at 4°C. CHI (EC 3.2.1.14) activity was determined according to the method of Abeles et al. (1971). One unit of CHI activity is defined as the amount of enzyme required to catalyze the production of 1  $\mu$ g *N*-acetyl-D-glucosamine per hour at 37°C. GLU (EC 3.2.1.58) activity was measured according to the method of Abeles et al. (1971). One unit of GLU activity is defined as the amount of enzyme catalyzing the formation of 1  $\mu$ M glucose equivalents in 1 h.

Membrane integrity was assayed by the method of Liu et al. (2007). The conidial suspensions were treated with the antagonist and stained with 5  $\mu$ M propidium iodide for 5 min at 30°C. The spores were observed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany) equipped with an individual fluorescein rhodamine filter set. The intracellular leakage of mycelia was determined according to the method of Liu et al. (2007). Soluble protein content was determined by the Bradford

(1976) method and soluble sugar content was estimated by the phenol-sulfuric acid method of Dubois et al. (1956). The results were expressed as mg/g wet weight of mycelia.

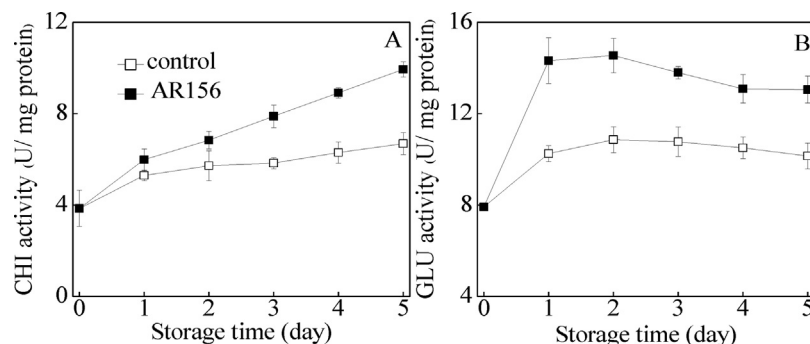
### 2.4. Statistical analysis

Each treatment was replicated three times and the experiment also repeated three times. All statistical analyses were performed with SPSS 11.0 (SPSS Inc., Chicago, IL, USA) for this experiment. Analysis of variance (ANOVA) was used to compare the means. Mean separations were performed using Duncan's multiple range tests. Differences at  $p < 0.05$  were considered to be significant.

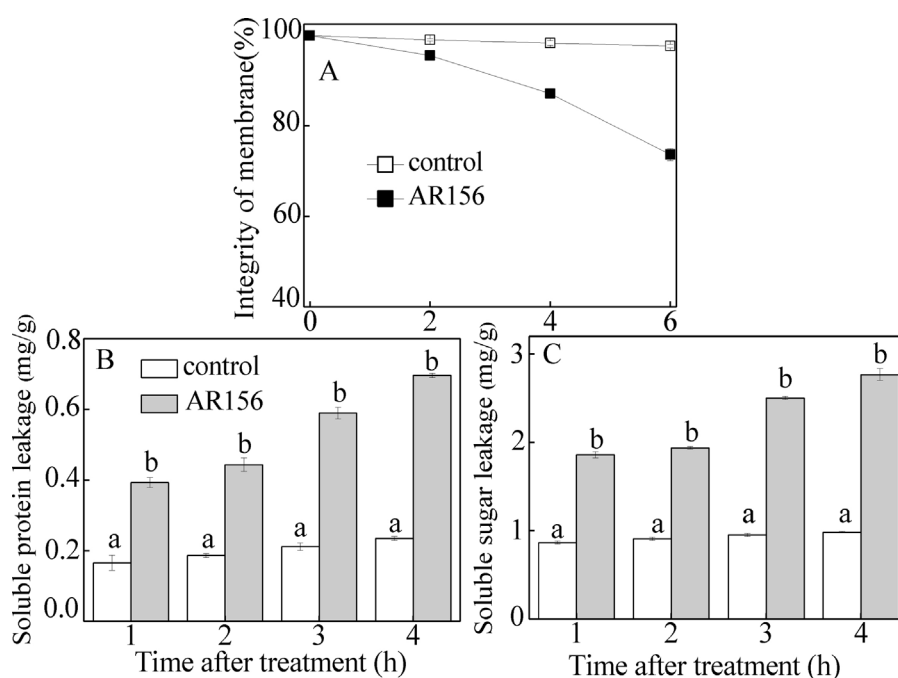
## 3. Results and discussion

The disease incidence and lesion diameter of blue mold decay caused by *P. expansum* in fruit treated with *B. cereus* AR156 were significantly ( $p < 0.05$ ) lower than those on the control after 5 days of incubation at 20°C. *B. cereus* AR156 treatment reduced decay incidence and lesion diameter by 79.3% and 32.2% compared with the control (Fig. 1A and B). This result suggests that *B. cereus* AR156 possesses strong antagonistic activity against the pathogen in sweet cherry fruit.

Induced resistance has been inferred to be one of the major mechanisms of biocontrol agents in inhibiting postharvest diseases of horticultural crops (Walters et al., 2005). The disease resistance in harvested fruit is associated with some inducible compounds including pathogenesis-related (PR) proteins. Among PR proteins, CHI and GLU play an important role in disease resistance of fruit. CHI has been proved to degrade chitin, which is the major component of pathogen cell walls. GLU can act indirectly by releasing oligosaccharides and eliciting defense reactions and then act synergistically with CHI to inhibit fungal growth



**Fig. 2.** Effect of *B. cereus* AR156 treatment on CHI (A) and GLU (B) activities in sweet cherry fruit inoculated with *P. expansum*. Data are expressed as the mean of triplicate samples. Vertical bars represent the standard errors of the means.



**Fig. 3.** Effects of *B. cereus* AR156 on plasma membrane integrity (A), protein (B) and sugar (C) leakage of *P. expansum* spores. Data are expressed as the mean of triplicate samples. Bars represent standard deviations of the means. Data in columns with the different letters are significantly different according to Duncan's multiple range tests at  $p < 0.05$ .

(Lee et al., 2006). Our results showed that the application of *B. cereus* AR156 resulted in a significant increase in the activities of CHI and GLU (Fig. 2). Induction of these two defensive enzymes by antagonists was also observed in peach and loquat fruit, which was correlated with increased disease resistance and reduced disease severity (Cao et al., 2008; Wang et al., 2013). Thus, the enhanced activities of these two defense-related enzymes could be one part of the mechanism by which *B. cereus* AR156 suppressed blue mold decay in sweet cherry fruit.

The plasma membrane plays a crucial role in maintaining fungal viability. Membrane damage can lead to the leakage of functional intracellular components such as proteins and sugars, thus weakens fungal viability (Wei et al., 2008). In this study, *B. cereus* AR156 treatment remarkably decreased the plasma membrane integrity of *P. expansum* spores, and increased leakage of proteins and sugars (Fig. 3), which suggested that *B. cereus* AR156 damaged plasma membranes of *P. expansum* spores. Zhou et al. (2011) also found that *B. subtilis* fmbj strongly induced morphological abnormalities and destroyed structure of hyphae and spores of *R. stolonifer*, which was correlated with reduced development of soft rot caused by the pathogen in harvested peach fruit. Therefore, our results suggest the direct fungitoxic activity against the pathogen could be another part of the mechanism by which *B. cereus* AR156 suppressed blue mold decay in sweet cherry fruit.

In conclusion, our results suggested that *B. cereus* AR156 could effectively inhibit blue mold decay caused by *P. expansum* in sweet cherry fruit, possibly by directly damaging fungal viability, and indirectly inducing disease resistance in the fruit.

## Acknowledgements

This study was supported by National Natural Science Foundation of China (No. 31172003 and 31301565), the National Natural Science Foundation of Shandong Province (ZR2012CQ009) and the Jiangsu Provincial Postgraduate Innovation Project of China (CXLX13\_266).

## References

- Abeles, F.B., Bosshart, R.P., Forrence, L.E., Habig, W.H., 1971. Preparation and purification of glucanase and chitinase from bean leaves. *Plant Physiol.* 47, 129–134.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle-dye binding. *Anal. Biochem.* 72, 248–254.
- Cao, J., Zhang, H.Y., Yang, Q.Y., Ren, R., 2013. Efficacy of *Pichia caribbica* in controlling blue mold rot and patulin degradation in apples. *Int. J. Food Microbiol.* 162, 167–173.
- Cao, S.F., Zheng, Y.H., Tang, S.S., Wang, K.T., 2008. Improved control of anthracnose rot in loquat fruit by a combination treatment of *Pichia membranifaciens* with  $\text{CaCl}_2$ . *Int. J. Food Microbiol.* 126, 216–220.
- Ceponis, M.J., Cappellini, R.A., Lightner, G.W., 1987. Disorders in sweet cherry and strawberry shipments to the New York market, 1972–1984. *Plant Dis.* 71, 472–475.
- Dubois, M., Gibbs, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric methods for the determination of sugars and related substances. *Anal. Chem.* 28, 350–352.
- Janisiewicz, W.J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. *Annu. Rev. Phytopathol.* 40, 411–441.
- Lee, J., Bricker, T.M., Lefevre, M., Pinson, S.R.M., Oard, J.H., 2006. Proteomic and genetic approaches to identifying defence-related proteins in rice challenged with the fungal pathogen *Rhizoctonia solani*. *Mol. Plant Pathol.* 7, 405–416.
- Liu, J., Tian, S.P., Meng, X.H., Xu, Y., 2007. Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. *Postharvest Biol. Technol.* 44, 300–306.
- Qin, G.Z., Tian, S.P., Xu, Y., Chan, Z.L., Li, B.Q., 2006. Combination of antagonistic yeasts with two food additives for control of brown rot caused by *Monilinia fructicola* on sweet cherry fruit. *J. Appl. Microbiol.* 100, 508–515.
- Sharma, R.R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: a review. *Biol. Control* 50, 205–221.
- Waewthongrak, W., Pisuchpen, S., Leelasuphakul, W., 2015. Effect of *Bacillus subtilis* and chitosan applications on green mold (*Penicillium digitatum* Sacc.) decay in citrus fruit. *Postharvest Biol. Technol.* 99, 44–49.
- Wang, X.L., Wang, J., Jin, P., Zheng, Y.H., 2013. Investigating the efficacy of *Bacillus subtilis* SM21 on controlling *Rhizopus* rot in peach fruit. *Int. J. Food Microbiol.* 164, 141–147.
- Walters, D., Walsh, D., Newton, A., Lyon, G., 2005. Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. *Phytopathol* 95, 1368–1373.
- Wei, M.K., Wu, Q.P., Huang, Q., Wu, J.L., Zhang, J.M., 2008. Plasma membrane damage to *Candida albicans* caused by chlorine dioxide ( $\text{ClO}_2$ ). *Lett. Appl. Microbiol.* 47, 67–73.
- Zhou, X.H., Lu, Z.X., Lv, F.X., Zhao, H.Z., Wang, Y., Bie, X.M., 2011. Antagonistic action of *Bacillus subtilis* strain fmbj on the postharvest pathogen *Rhizopus stolonifer*. *J. Food Sci.* 76, 254–259.